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Mycotoxins Produced from Fungi Isolated from Foodstuffs and Soil: Comparison of Toxicity in Fibroblasts and Rat Feeding Tests

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Thirty-nine isolates of fungi obtained from foodstuffs and soil samples from various parts of the world have been identified. The isolates were grown on a solid rice medium, and extracts were prepared with 50% aqueous methanol. The extracts were examined for toxicity in the following systems: (i) cytotoxicity to cultured normal human diploid skin fibroblasts (proliferating and nonproliferating) and mouse fibroblasts; (ii) skin toxicity after topical application on rats; and (iii) rat feeding tests in which rats were examined for death, overt pathological effects including congestion and hemorrhage of tissues, weight loss, food refusal, and uterine growth. Sixteen culture extracts were highly toxic as indicated by death, congestion and hemorrhage of tissues, and net weight loss. One half of the isolates were highly cytotoxic (50% lethal concentration, 0.01 to 5 μg/ml) as indicated by the ability to cause death and disintegration of 3T3 Swiss mouse fibroblasts and human diploid skin fibroblasts during 3 to 4 days in culture. The remainder were moderately cytotoxic (50% lethal concentration, 5 to 250 μg/ml). Four culture extracts were highly toxic by some clinical criteria but did not cause congestion and hemorrhage of tissues and were weakly cytotoxic (50% lethal concentration, 250 to 5,000 μg/ml). Six culture extracts exhibited moderate toxicity (weight loss only) and low cytotoxicity (50% lethal concentration, 3,000 to 50,000 μg/ml). Four culture extracts caused uterine enlargement as the major clinical sign, suggesting the presence of zearalenone. Eleven culture extracts were weakly cytotoxic and caused no major clinical signs, except skin toxicity in two extracts. Cytotoxicity values of fungal extracts obtained with cultured cells, the simplest and least expensive assay used in this study, correlated well with weight loss and food refusal observed in rat feeding studies, but not with skin toxicity or uterine growth. Extracts of several fungal isolates inhibited cell proliferation without killing cells, a characteristic not observed with any identified toxin studied in this laboratory.

Various types of fungi, particularly *Fusarium* spp., are common and widespread throughout the environment. There is a long-standing history of toxicosis associated with the consumption of fungus-infected cereal grains by people or their domestic animals during periods of adverse economic conditions (28). Detailed studies of some of these outbreaks of toxicosis have led to the identification of a large series of mycotoxins including moniliformin, zearalenone, the trichothecenes, and the macrocyclic trichothecenes (1a, 13, 23, 29).

A variety of physical and bioassay methods have been developed to study the mycotoxins of *Fusarium* spp. (17, 22). Thin-layer chromatography is very specific and readily adapted to screening large numbers of samples, but it lacks sensitivity. Liquid chromatographic methods also lack sensitivity and are not practical for screening large numbers of samples. Radioimmunoassay and polarographic methods have been developed for some individual toxins, but they are not readily applied to screening complex mixtures with unknown toxins of closely related structure. Gas-liquid chromatographic methods with quantitation by flame ionization, electron capture, or mass spectrometric detection are very specific and are the most sensitive methods available, but they are not easily adapted to screening large numbers of samples. Several methods have been developed for bioassay of *Fusarium* spp. toxins with various animals (5, 7, 8, 11, 21, 25, 26, 30), cultured tumor cells (20, 24), or cultured normal human and mouse fibroblasts (1). Bioassay with cultured fibroblasts (1) is readily adapted to the screening of large numbers of samples, and it possesses the ability to detect the presence of toxins of unknown structure, but it lacks selectivity. The sensitivity varies from toxin to toxin, but in the case of T-2 toxin with 3T3 mouse fibroblasts (1), it is comparable to the most sensitive physical method (gas-liquid chromatography–mass spectrometry).

Although most studies on fungal toxicosis have been carried out in response to unintentional poisoning of domestic animals by one or a limited number of fungal strains, some studies have been carried out to examine larger numbers of food-contaminating fungi in experimental systems that permit more detailed study and comparison between strains (2). Saito et al. (20) used cultured human ovarian carcinoma cells and mice to screen more than 800 isolates of food-contaminating fungi collected in two localities (mountain farm and seaside village) in Japan. Abbas et al. (1) reported the toxicity of five *Fusarium* sp. culture extracts from a wider range of sources with a mouse fibroblast bioassay. In view of the limited number of studies using comparable assay systems on toxin-producing fungi from widely differing parts of the world, we undertook the present comparison of 39 fungal isolates from widely separated parts of the world.

MATERIALS AND METHODS

Fungus cultures. The sources and types of fungi used in these studies are given in Table 1. Species identification was made by the donor, except the following strains, which were identified by C. E. Windels by the method of Nelson et al. (15): *Fusarium acuminatum* FS.732, *F. acuminatum* NF.5100, *F. avenaceum* FS.737, *F. culmorum* HM-8, *F. moniliforme* var. *subglutinans* 7, *F. moniliforme* 248, *F. roseum* subsp. *graminearum* 23, *F. roseum* subsp. *gramin-*

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TABLE 1. Fungal strains used in this study and their sources

Fungus	Code no.	Source	Country of origin	Donor[a]
Fusarium spp.				
F. acuminatum	FS.732	Barley	United States (Alaska)	A
F. acuminatum	NF.5100	Corn	China	B
F. avenaceum	FS.737	Barley	United States (Alaska)	A
F. avenaceum	3	Wheat plant	Norway	C
F. camptoceras	NF.2833	Corn	China	B
F. culmorum	1	Barley plant	Norway	C
F. culmorum	HM-8	Wheat	England	D
F. graminearum	2	Barley kernels	Norway	C
F. graminearum	NF.1432	Wheat	China	B
F. graminearum	NF.1436	Wheat	China	B
F. graminearum	NF.0996	Corn	China	B
F. moniliforme	NF.1439	Corn	China	B
F. moniliforme	248	Wheat	Egypt	E
F. moniliforme var. *subglutinans*	7	Wheat kernels	United States (Minnesota)	A
F. oxysporum	1a	Soil	Egypt	E
F. oxysporum	1b	Soil	Egypt	E
F. oxysporum	1c	Soil	Egypt	E
F. poae	4	Wheat kernels	Norway	C
F. poae	NF.5098	Corn	China	B
F. roseum subsp. *gibbosum*		Animal feed	United States (Minnesota)	A
F. roseum subsp. *graminearum*	23	Wheat	United States (Minnesota)	A
F. roseum subsp. *graminearum*	A-2-2	Oats	United States (Alaska)	A
F. roseum subsp. *graminearum*	25	Soil	United States (Minnesota)	A
F. roseum subsp. *graminearum*	27	Wheat	United States (Minnesota)	A
F. roseum subsp. *graminearum*	28	Wheat	United States (Minnesota)	A
F. roseum subsp. *graminearum*	29	Wheat	United States (Minnesota)	A
F. semitectum	NF.5099	Corn	China	B
F. solani	IF	Soil	Egypt	E
F. sporotrichioides	NF.2832	Corn	China	B
F. sporotrichioides	33-1972	Corn	Russia	F
F. sporotrichioides	33-1979	Corn	Russia	F
F. tricinctum	NRRL-3299	Soil	United States	G
Gerlachia spp.				
G. nivalis	5	Wheat kernels	Norway	C
G. nivalis	6	Oat kernels	Norway	C
G. nivalis	7	Timothy grass	Norway	C
Myrothecium spp.				
M. verrucaria	C.2561	Pure culture	England	D
M. verrucaria	C.2562	Pure culture	England	D
Stachybotrys spp.				
S. atra	82-x	Pure culture	Belgium	H
S. bisby	C.2560	Pure culture	England	D
Papulospora sp.	4	Wheat kernels	United States	A

[a] Isolates of fungi were obtained from the following donors: A, this laboratory (C.J.M.), 1981 to 1983; B, I. Lu Shih (1981), The Institute of Microbiology, Academy of Science, Beijing, People's Republic of China; C, Leif Sundhe (1983), Norwegian Plant Protection Institute, Department of Plant Pathology, Boks 10, N-1432 AS-NLH, Norway; D, J. Lacy (1982), Rothamsted Experimental Station, Harpenden, Hertfordshire., AL5 2JQ, England; E, Ismail El-kady (1981–1982) Department of Botany, Assiut University, Assiut, Egypt; F, M. Palyusik, Budapest, Hungary; G, Northern Regional Research Laboratory, Peoria, Ill.; H, Sarkeres Ztur (1983) Budapest, Hungary.

earum A-2-2, *F. roseum* subsp. *graminearum* 27, *F. roseum* subsp. *graminearum* 29, and *Papulospora* sp. 4. Stock cultures of these isolates were maintained in moist, autoclaved soil stored at −15°C.

Mammalian cell cultures. Human diploid fibroblasts (GM3349, from an apparently normal 10-year-old male by skin biopsy, passage 14) were obtained from the Institute for Medical Research, Camden, N.J. 3T3 Swiss mouse fibroblasts were obtained from R. W. Holley, Salk Institute, La Jolla, Calif. Cell lines were maintained by subculturing into 10% (vol/vol) calf serum (GIBCO Laboratories, Grand Island, N.Y.) in Dulbecco modified Eagle medium (GIBCO) with transfer achieved with 0.05% (wt/vol) trypsin in medium to remove cells. GM3349 cells were subcultured at 0.5 density, and 3T3 cells were cultured at 0.1 density in 10-mm plastic culture dishes at 37°C in a humidified atmosphere

containing 15% CO_2 in air. Long-term storage was in liquid nitrogen in medium containing 10% dimethyl sulfoxide and 20% calf serum.

Mycotoxin standards. Diacetoxyscirpenol was purchased in analytically pure form from Sigma Chemical Co., St. Louis, Mo. The following toxins were produced and purified in this laboratory by the indicated method: T-2 toxin (3), neosolaniol (4), HT-2 toxin (4), zearalenone (14), acetyl T-2 (16), 4-deacetylneosolaniol (TMR-1) (32), 8-acetyl T-2 tetraol (TMR-2) (32), and 15-deacetylneosolaniol (N-1) (6). Deoxynivalenol, nivalenol, 7-hydroxydiacetoxyscirpenol, and fusarenone-x were prepared in this laboratory by unpublished methods. Moniliformin was a gift from H. Burmeister, U.S. Department of Agriculture, Northern Regional Research Center, Peoria, Ill. 3-Acetyl-deoxynivalenol was the gift of J. D. Miller, Canada. 3'-Hydroxy T-2 (Tc-1) and 3'-hydroxy HT-2 (Tc-3) were the gifts of T. Yoshizawa, Department of Food Science, Faculty of Agriculture, Kagawa University, Kaguwa-kun, Japan.

Preparation of fungus extracts. Crude fungus culture extracts were prepared by a modification of a method reported by Eugenio et al. (6). Briefly 200 g of long-grain polished rice and 120 ml of distilled water were allowed to stand for 1 h in a 1-liter flask, shaken, cotton stoppered, and autoclaved for 60 min at 121°C and 15 lb (ca. 6.8 kg) of pressure. After 24 h at room temperature the flasks were reautoclaved, shaken, and inoculated when cool from stock cultures maintained in sterile soil. The flasks were incubated at room temperature (22 to 25°C) with daily shaking for the first few days to permit the fungus to uniformly penetrate the rice. The cultures were then incubated for 2 weeks at 25 to 27°C, followed by 2 weeks at 10°C. The mass of fungus-invaded rice was broken up, transferred to a screen-bottomed tray, and allowed to air dry in a ventilated hood. The moldy rice was ground to the consistency of flour in a coffee grinder in a fume hood to prevent human exposure to aerosols during the grinding process. Ground moldy rice (10 g) was extracted three times for 1 h at room temperature with a total of 200 ml of 50% aqueous methanol. The combined extracts were filtered through Whatman no. 4 filter paper and concentrated to 10 ml on a rotary evaporator. Half of this material was stored to be used directly for fibroblast cytotoxicity assays, and the remainder was evaporated to dryness on the rotary evaporator. The residue was dissolved in 1 ml of acetone for use in rat skin toxicity testing.

Rat feeding toxicity study. Twenty-day-old virgin female Wistar rats (Holtzman Supply Co., Madison, Wis.) were housed in individual cages and fed a 1:1 mixture of ground moldy rice prepared as described above and ground complete rat chow (Ralston-Purina) in powder form. The animals and feed were weighed at the beginning and end of the experiment. Three rats were used for each fungal isolate as well as three control rats that received chow only. They were observed frequently for 5 days, and major symptoms and death were recorded. Surviving rats were sacrificed by diethyl ether inhalation and examined for pathological changes in the stomach, lungs, liver, heart, uterus, fat bodies, inner skin layer, and intestines. Uterine weight was determined. The effects on the microbial flora of the rats were not investigated.

Rat skin toxicity study. Virgin female Wistar rats (approximately 70 g) were shaved with electric clippers in a 2- by 3-cm area on the back. Pairs of rats each received the extract prepared from 2.5 g of moldy rice applied in 500 μl of acetone with a micropipette to the shaved area. Control rats received pure acetone. The rats were placed in individual cages and provided food and water ad libitum. The rats were observed frequently for 48 h after dosing and then sacrificed by diethyl ether inhalation. The degree of toxicity was as follows: D, death; +, definite skin toxicity symptoms in the shaved area; −, no positive evidence of toxic effect.

Fibroblast cytotoxicity determinations. Cytotoxicity assays were carried out essentially as described elsewhere (1) with Swiss mouse 3T3 fibroblasts and human diploid skin GM3349 fibroblasts in 96-well plastic culture trays (3042 Micro-Test II; Falcon Plastics, Oxnard, Calif.) Assays were carried out in triplicate for extracts from each fungal isolate in an initial range-finding assay with 3T3 cells and subsequently in triplicate for each of 3T3 and GM3349 cells with a narrower range of concentrations. The initial culture extracts represented 1,000 μg of moldy rice equivalent per ml. Serial dilutions in culture medium were carried out with a pipetting aid and sterile plastic tips before the addition of the cell inoculum in medium containing calf serum. Because the culture medium contained antibiotics (penicillin and streptomycin), it was not necessary to sterilize fungal extracts before assay. The cultures were incubated until the optimal cell density was achieved, fixed with Formalin, and stained with Giemsa. The inoculum size used permitted approximately four doublings by 3T3 cells and approximately one doubling by GM3349 cells. The approximate concentration of toxin resulting in one half the control cell density (LC_{50}) was estimated visually.

Qualitative detection of toxins in culture extracts. Cultures were extracted and analyzed by a modification of the methods described in detail by Yoshizawa et al. (31). Briefly, thin-layer chromatography was carried out on precoated (0.25-mm-thick) silica gel 60 plates without fluorescent indicator (E. Merck, Darmstadt, Federal Republic of Germany) in the following solvent systems: (i) chloroform-methanol, 9:1 (for general mycotoxin studies); (ii) toluene-acetone-methanol, 5:3:2 (for moniliformin); (iii) ethyl acetate-toluene, 3:1 (for zearalenone and 3-acetoxydeoxynivalenol); and (iv) chloroform-methanol, 4:1 (for polar trichothecenes). The following methods were used to detect trichothecenes, nontrichothecenes, and zearalenone. (i) Thin-layer chromatograms were sprayed with sulfuric acid-methanol (20:80), heated at 110°C for 10 min, and examined under longwave UV light (356 nm) for trichothecenes (gray fluorescence) and zearalenone (orange). (ii) Thin-layer chromatograms were sprayed with *p*-anisaldehyde freshly prepared as described by Pathre et al. (18) and heated for 10 min at 110°C. Trichothecene toxins related to T-2 gave brownish to violet spots, 8-keto-trichothecenes gave canary yellow spots, and zearalenone gave an orange-yellow spot. (iii) Thin-layer chromatograms were treated by the method of Kato and Takitani (10) for detection of trichothecenes as blue spots. The plates were dipped in a 3% (wt/vol) solution of 4-(*p*-nitrobenzyl)pyridine in chloroform-carbon tetrachloride (2:3), dried, heated for 30 min at 110°C, cooled, and dipped in a 10% (vol/vol) solution of tetraethylene-pentamine in

TABLE 2. Comparison of cytostatic and cytotoxic activities of culture extracts with human skin fibroblasts (GM3349)

Fungus	LC_{50} (μg of moldy rice equivalent per ml)	
	Cytostatic effect	Cytotoxic effect
F. acuminatum FS.732	5,000	50,000
F. avenaceum FS.737	5,000	35,000
F. culmorum HM-8	2,000	35,000
Papulospora sp. 4	2,000	20,000

chloroform-carbon tetrachloride (2:3). (iv) Thin-layer chromatograms were treated by the methods of Kamimura et al. (9) and Rabie et al. (19) for the detection of moniliformin as a red-brown spot on thin-layer chromatograms after spraying with 0.32% (wt/vol) 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid followed by heating for 10 min at 110°C.

RESULTS

Thirty-nine cultures of fungal isolates (Table 1) were prepared and tested for toxicity in the following three assay systems: (i) cytotoxicity in cultures of 3T3 mouse fibroblasts and human diploid skin fibroblasts (line GM3349), which yield quantitative or semiquantitative measures of toxicity of culture extracts (1); (ii) rat skin toxicity testing of culture extracts, which measures transdermal absorption and toxicity in the form of skin necrosis or death; and (iii) a rat feeding test on unextracted cultures with observation for the following: weight gain; food refusal; congestion and hemorrhaging of tissues due to break down of capillary vessel walls in the nose, lungs, intestine, liver, and stomach; appearance of fat bodies in tissues; uterine enlargement (an indication of the production of zearelanone [12]); and death.

Eight cultures (*F. acuminatum* NF.5100, *Fusarium poae* NF.5098, *F. sporotrichioides* 33-1972; *F. sporotrichioides* 33-1979, *F. tricinctum* NRRL-3299, *Stachybotrys atra* 82-x, *Myrothecium verrucaria* C.2561, and *M. verrucaria* C.2562) were highly toxic by each of the following criteria: marked food refusal; decreased weight gain or weight loss; death; congestion and hemorrhaging of lungs, intestine, liver, and stomach; reduced fat bodies among the tissues; and nasal hemorrhaging. The culture extracts induced skin necrosis and cytotoxicity at low concentrations (LC_{50} values of 0.01 to 5 μg/ml in 3T3 and 0.01 to 100 μg/ml in GM3349). No uterine enlargement was observed.

An additional eight cultures exhibited a similar degree of toxicity in the rat feeding test, but culture extracts exhibited weaker cytotoxicity (LC_{50} values of 5 to 750 μg/ml with 3T3 and 2,500 to 35,000 μg/ml with GM3349). Extracts of five of these cultures (*Fusarium culmorum* 1, *F. graminearum* 2, *F. graminearum* NF.1436, *F. poae* 4, *Gerlachia nivalis* 7) induced skin necrosis, and three (*F. moniliforme* 248, *F. moniliforme* var. *subglutinans* 7, and *F. roseum* subsp. *graminearum* A-2-2) did not. The five cultures that induced skin necrosis also caused a breakdown of capillaries in the inner skin layer.

Four cultures (*F. avenaceum* 3, *F. camptoceras* NF.2833, *F. culmorum* HM-8, and *F. roseum* subsp. *graminearum* 23) caused weight loss, food refusal, reduction of fat bodies, and other major clinical signs of toxicity, but they induced no congestion of tissues or breakdown of capillary tissues in the inner skin layer. Also, culture extracts were weakly cytotoxic (LC_{50} values in the range of 1,000 to 10,000 μg/ml for 3T3 and 10,000 to 35,000 μg/ml for GM3349).

Six cultures (*F. avenaceum*, *F. oxysporum* 1a, *F. oxysporum* 1b, *F. oxysporum* 1c, *F. solani* 1f, *S. bisby* C.2560) exhibited a degree of toxicity as indicated by weight loss and reduction of fat bodies, but no uterine enlargement or skin necrosis. Extracts of these cultures exhibited moderate to weak cytotoxicity (LC_{50} values in the range 100 to 5,000 μg/ml with 3T3 and 5,000 to 50,000 μg/ml for GM3349).

Four cultures (*F. graminearum* NF.1432, *F. roseum* subsp. *graminearum* 23, *F. roseum* subsp. *graminearum* 28, and *F. roseum* subsp. *graminearum* 29) induced uterine enlargement as the major clinical sign. The observed degree of uterine enlargement is consistent with production of zearalenone (12, 29).

Twelve cultures (*F. graminearum* NF.0996; *F. roseum* subsp. *gibbosum*; *F. acuminatum* FS732; *F. moniliforme* NF.1439; *F. graminearum* NF.1432; *F. semitectum* NF.5099; *F. roseum* subsp. *graminearum* 27, 28, and 29; *F. sporotrichioides* NF.2832; *G. nivalis* 5 and 6) caused no major clinical signs of toxicity, and only extracts of the first two strains listed above caused skin toxicity.

TABLE 3. Toxins detected in extracts of selected fungal isolates

Fungus	Toxins[a]
F. acuminatum FS.732	Unknown metabolites[b]
F. acuminatum NF.5100	T-2, neosolaniol, 3′-hydroxy T-2, HT-2, 4-deacetylneosolaniol, 8-acetyl T-2 tetraol, diacetoxyscirpenol, 15-deacetylneosolaniol, T-2 tetraol, plus several unknown trichothecenes[b]
F. avenaceum FS.737	Unknown metabolites
F. avenaceum 3	Deoxynivalenol, fusarenone-X
F. camptoceras NF.2833	Unknown trichothecenes[b]
F. culmorum 1	Deoxynivalenol, 3-acetoxydeoxynivalenol, zearalenone[b]
F. culmorum HM-8	Unknown metabolites[b]
F. graminearum 2	Deoxynivalenol, 3-acetoxydeoxynivalenol, zearalenone[b]
F. graminearum NF.1432	Deoxynivalenol, zearalenone Unknown metabolites
F. moniliforme 248	
F. moniliforme var. *subglutinans* 7	Moniliformin, unknown metabolites[b]
F. poae 4	Nivalenol, fusarenone-X
F. poae NF.5098	T-2, neosolaniol, HT-2, 4-deacetylneosolaniol, 8-acetyl T-2 tetraol, T-2 tetraol, several unknown trichothecenes[b]
F. roseum subsp. *gibbosum*[c]	Monacetoxyscirpenol and isomers, diacetoxyscirpenol and isomers, triacetoxyscirpenol, scripentriol, zearalenone[b]
F. roseum subsp. *graminearum* 23[d]	Deoxynivalenol, zearalenone
F. roseum subsp. *graminearum* A-2-2[d]	7-Hydroxydiacetoxyscirpenol, T-2 tetraol, diacetoxyscirpenol, unknown metabolites[b]
F. sporotrichioides 33-1972	T-2, HT-2, neosolaniol, 4-deacetylneosolaniol, 8-acetyl T-2 tetraol, T-2 tetraol, several unknown trichothecenes
F. sporotrichioides 33-1979	T-2, HT-2, neosolaniol, 4-deacetylneosolaniol, 8-acetyl T-2 tetraol, T-2 tetraol, several unknown trichothecenes
F. trincinctum NRRL-3299[e]	T-2, HT-2, neosolaniol, 8-acetylneosolaniol, T-2 tetraol, 4-deacetylneosolaniol, 8-acetyl T-2 tetraol, deacetyl HT-2, and acetyl T-2[b]
G. nivalis 7	Deoxynivalenol, fusarenone-X

[a] Unknown metabolites with chromatographic properties characteristic of trichothecenes that stain blue after treatment by the method of Kato and Takitani (10) are considered unknown trichothecenes.

[b] These identifications were confirmed by gas chromatographic-mass spectrometric analysis.

[c] This isolate was analyzed by X. Zhu in this laboratory.

[d] This isolate was analyzed by Yin Won Lee in this laboratory.

[e] This isolate was analyzed by S. T. Swanson in this laboratory.

The cytotoxicity assay is carried out with the human diploid skin fibroblast line GM3349 with an initial cell density of about one-half the final saturating cell density at the end of the assay. Consequently, it is possible to simultaneously observe cytostatic activity (which prevents cell division) as well as cytotoxicity (cell killing in the presence or absence of cell division). Cytostatic activity is characterized by a low cell density with the dilution endpoint occurring where the cell density is at control levels. Cytotoxic activity is characterized by the absence of all or most cells at the end of the assay. Four culture extracts (from *F. culmorum* HM-8, *F. avenaceum* FS.737, *F. acuminatum* FS.732, and *Papulospora* sp. 4) exhibited cytostatic as well as cytotoxic activity (Table 2). Possibly other cultures contain this activity, but it is masked by cytotoxicity at equal or higher dilution.

Extracts of selected cultures were examined qualitatively for the presence of trichothecenes and structurally related metabolites, zearalenone and moniliformin, by thin-layer chromatography (Table 3). Mycotoxins were identified by cochromatography with authentic standards in at least two solvent systems and by identical color reactions of the spots after treatment with *p*-anisaldehyde reagent, 20% (vol/vol) methanolic sulfuric acid, or with the reagents of Kato and Takitani (10). The latter system is more specific for trichothecenes because they detect the epoxide group present in this class of toxins.

DISCUSSION

Previous studies of the toxicity of fungal isolates and culture extracts prepared from them have either focused on isolated outbreaks of toxicity in humans or domestic animals due to one or a limited number of fungal species or screened large numbers of isolates from a closely restricted geographical area. In the present study isolates from widely separated geographical areas were examined. The areas include extreme northern latitudes (Alaska, Norway), temperate areas (United States, China, England, Russia, Belgium), and southern latitudes (Egypt). In addition, a quantitative assay was used, permitting facile comparison of the toxicity of extracts from various strains. In animal feeding studies the animals were fed ground cultures of fungi on rice in their diet to provide a good model of the mycotoxicosis associated with consumption of moldy animal feed. The methods used in the study were effective at predicting which fungal isolates contain toxins and to identify isolates with unusual toxicological properties. All of the 16 highly toxic cultures identified in the rat feeding tests produce trichothecene toxins, except *F. moniliforme* 248 and *F. moniliforme* var. *subglutinans* 7 (Table 3 and unpublished results).

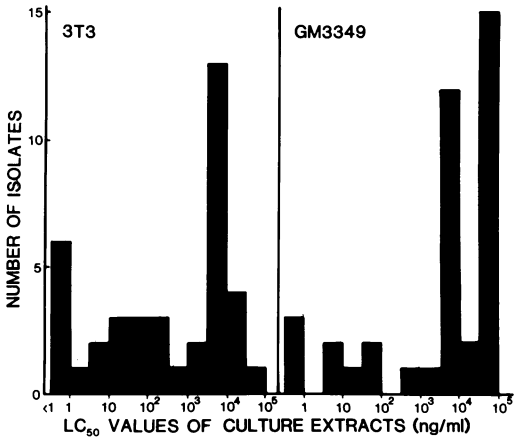

FIG. 1. Distribution of cytotoxicity values obtained with 3T3 mouse fibroblasts and *GM3349* human skin fibroblasts in culture.

Fungal extracts that exhibited toxic effects in rats (local necrosis, death) after skin applications also exhibited strongly toxic effects in the rat feeding test and the fibroblast cytotoxicity assay. However, many extracts that were highly toxic by other criteria (e.g., congestion and hemorrhage of tissues, food refusal, etc.) exhibited weak or undetectable toxicity in the skin test. This may be due to small sample size, but another explanation is lack of transcutaneous absorption due to suboptimal polarity and solubility characteristics. The latter explanation is supported by the results of other studies (1a, 13, 16, 23).

A comparison of relative cytotoxicity values observed for extracts from the various isolates with both mouse and human fibroblasts (Fig. 1) indicates that the distributions are nonuniform (i.e., multiphasic), consistent with there being more than one class of toxin producers. The different subgroups probably result from different types and possibly different classes of toxins being produced, because different amounts of the same toxins would be expected to give a more uniform distribution of toxicities. The known toxins produced by *Fusarium* spp. and other species in this study can be classified into three groups based on chemical structures (28): macrocyclic trichothecenes, nonmacrocyclic trichothecenes, and nontrichothecene toxins such as zearalenone and moniliformin. Examination of the cytotoxicity values of purified toxins in 3T3 and GM3349 cell systems (1; unpublished data) indicates that macrocyclic trichothecenes and T-2 are highly cytotoxic, whereas nonmacrocyclic trichothecenes except T-2 are moderately cytotoxic, and the nontrichothecene toxins (e.g., moniliformin, zearalenone) exhibit little or no cytotoxicity with either cell line. Selected fungal isolates were examined qualitatively for the types of toxins present in extracts (Table 3) to determine whether the types of known toxins produced could explain the distribution of cytotoxicity values. Many of the isolates examined in Table 3 produce unknown metabolites with some of the structural features characteristic of trichothecene toxins. Better knowledge of the full range of toxic metabolites produced by *Fusarium* spp. will be required before the cytotoxicity values of the complex mixtures of toxins produced can be understood.

There is currently much interest (27) in developing cellular systems for toxicity testing because they are usually less expensive, use less sample, are more quantitative, are more rapid, and avoid ethical considerations inherent in whole animal testing. The cytotoxicity assays employed in this study possess these advantages, but it should be noted that the animal feeding studies provide different and complementary information. Many of the various activities detected in whole animals (e.g., congestion and hemorrhaging of tissues) cannot be effectively modeled in cell culture. Ultimately the final assessment of toxicity of a fungal extract or any other agent must be carried out in intact animals.

The present study allows an examination of the effectiveness of the cytotoxicity assays as predictors of toxicity in the whole animal. The average weight gain or loss (Table 4) was compared (analysis of variance) with the LC_{50} values of extracts on 3T3 cells and GM3349 (Table 5) with and without logarithmic (base 10) transformation. Cytotoxicity values obtained with 3T3 cells correlate well with weight loss ($r = 0.63$, $F = 24.7$, $P = 1.5 \times 10^{-5}$), and logarithmic transforma-

TABLE 4. Toxicity of fungal isolates in rats fed a mixture of moldy rice and chow

Fungus	Average wt gain (g)	Food consumption (g)	Uterine wet wt (% of control)[a]	Congestion and hemorrhage of tissues[b]
Control	28.6 ± 4.3	39.6 ± 5.3	100	–
F. acuminatum FS.732	0.3 ± 2.3	9.3 ± 3.5	50 ± 44	–
F. acuminatum NF.5100	−17.7 ± 0.9	3.0 ± 1.0	36 ± 19	+, D
F. avenaceum FS.737	−2.0 ± 3.0	13.3 ± 5.2	61 ± 9	–
F. avenaceum 3	10.7 ± 3.7	7.7 ± 0.7	NT[c]	–
F. camptoceras NF.2833	−15.0 ± 0	2.3 ± 1.4	35 ± 3	–
F. culmorum 1	−16.3 ± 1.4	3.7 ± 1.3	NT	+, D
F. culmorum HM-8	−8.3 ± 1.3	7.3 ± 2.9	NT	–
F. graminearum 2	−16.3 ± 0.9	3.3 ± 1.4	NT	+, D
F. graminearum NF.1432	3.3 ± 0.9	17.3 ± 5.7	175 ± 32	–
F. graminearum NF.1436	−2.7 ± 1.2	11.0 ± 1.2	64 ± 7	+, D
F. graminearum NF.0996	7.0 ± 2.5	25.3 ± 1.4	96 ± 11	–
F. moniliforme 248	−11.0 ± 2.5	4.0 ± 0.6	32 ± 2	+, D
F. moniliforme NF.1439	3.7 ± 3.9	11.3 ± 2.7	35 ± 13	–
F. moniliforme NF.1439	3.7 ± 3.9	11.3 ± 2.7	35 ± 13	–
F. moniliforme var. *subglutinans* 7	−10.3 ± 2.3	6.0 ± 0.6	101 ± 0	+, D
F. oxysporum 1a	−7.3 ± 0.3	8.0 ± 1.5	44 ± 0	–
F. oxysporum 1b	−8.3 ± 0.3	9.0 ± 0.6	73 ± 2	–
F. oxysporum 1c	−8.3 ± 3.8	8.3 ± 2.2	57 ± 1	–
F. poae 4	−14.0 ± 1.5	3.7 ± 1.2	NT	+, D
F. poae NF.5098	−17.7 ± 1.2	4.3 ± 3.4	69 ± 29	+, D
F. roseum subsp. *gibbosum*	7.0 ± 1.2	22.0 ± 5.6	29 ± 3	–
F. roseum subsp. *graminearum* 23	−11.7 ± 6.1	3.0 ± 1.2	364 ± 155	–
F. roseum subsp. *graminearum* A-2-2	14.7 ± 1.2	2.3 ± 0.9	44 ± 11	–
F. roseum subsp. *graminearum* 27	5.0 ± 2.0	28.7 ± 4.4	124 ± 28	–
F. roseum subsp. *graminearum* 28	3.33 ± 0.9	25.3 ± 2.9	549 ± 83	–
F. roseum subsp. *graminearum* 29	10.0 ± 1.5	33.3 ± 2.8	160 ± 25	–
F. semitectum NF.5099	12.3 ± 2.7	29.3 ± 0.9	95 ± 18	–
F. solani If	−4.0 ± 1.2	8.7 ± 2.9	61 ± 3	–
F. sporotrichiodes NF.2832	15.3 ± 4.8	33.6 ± 6.0	46 ± 12	–
F. sporotrichiodes 33-1972	−10.0 ± 2.1	4.7 ± 0.3	49 ± 11	+, D
F. sporotrichiodes 33-1979	−17.0 ± 2.6	3.0 ± 0.6	45 ± 5	+, D
F. tricinctum NRRL-3299	−13.7 ± 1.4	12.7 ± 4.9	48 ± 9	+, D
G. nivalis 5	10.0 ± 7.5	30.7 ± 10.3	NT	–
G. nivalis 6	14.0 ± 2.6	37.7 ± 3.3	NT	–
G. nivalis 7	1.3 ± 3.3	12.3 ± 4.7	NT	+, D
M. verrucaria C.2561	−18.7 ± 0.7	4.3 ± 2.2	60 ± 3	+, D
M. verrucaria C.2562	−18.7 ± 0.9	6.3 ± 1.4	65 ± 2	+, D
S. atra 82-X	−17.3 ± 1.2	1.7 ± 0.3	NT	+, D
S. bisby C.-2560	−0.7 ± 2.3	12.3 ± 5.4	47 ± 4	–
Papulospora sp. 4	10.3 ± 2.6	20.3 ± 3.3	63 ± 10	–

[a] The average uterine wet weight at the end of the experiment was 96.0 ± 18.5 mg.
[b] +, Definite detectable toxic effect; –, no detectable toxic effect; D, death.
[c] NT, Not tested.

tion slightly improves the correlation ($r = 0.66$, $F = 28.9$, $P = 4 \times 10^{-6}$). Scattergrams comparing mean weight gain or loss with cytotoxicity values of extracts on 3T3 cells are given (Fig. 2) with and without logarithmic transformation. Cytotoxicity values obtained with GM3349 human skin fibroblasts correlate better with weight loss ($r = 0.69$, $F = 34.0$, $P = 10^{-6}$), but logarithmic transformation does not improve the correlation ($r = 0.58$, $F = 19.0$, $P = 10^{-4}$). The cytotoxicity values obtained with 3T3 cells also correlate well with food refusal activity (i.e., reduction in food consumption from control values) ($r = 0.74$, $F = 21.1$, $P = 5 \times 10^{-5}$), and logarithmic transformation has little effect on the correlation ($r = 0.62$, $F = 23.0$, $P = 2.7 \times 10^{-5}$). Cytotoxicity values obtained with GM3349 also correlated well with food refusal activity ($r = 0.68$, $F = 31.2$, $P = 2 \times 10^{-6}$), but logarithmic transformation resulted in poorer correlation ($r = 0.47$, $F = 10.4$, $P = 2.6 \times 10^{-3}$). Uterine weight gain

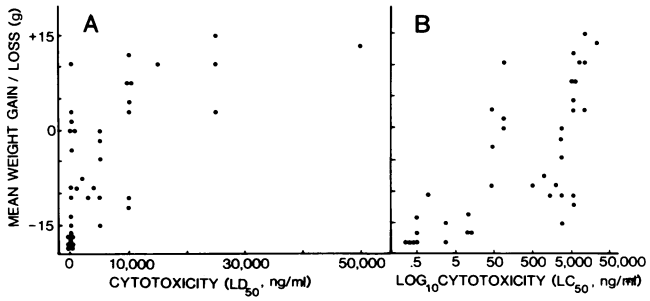

FIG. 2. Scattergrams comparing the distribution of mean weight gain or loss values obtained in feeding tests with cytotoxicity values observed with fungal extracts on 3T3 mouse fibroblasts. The distributions are compared with cytotoxicity values plotted on (A) a linear scale or (B) a logarithmic scale.

TABLE 5. Toxicity of extracts of fungal isolates cultured on rice with cultured mouse 3T3 and human GM3349 fibroblasts and with rats after direct application to the skin

Fungus	Cytotoxicity (LC_{50}, μg of moldy rice extracted per ml)		Rat skin toxicity[a]
	3T3	GM3349	
F. acuminatum FS.732	5,000	50,000	–
F. acuminatum NF.5100	0.75	5	+, D
F. avenaceum FS.737	5,000	35,000	–
F. avenaceum 3	5,000	10,000	+
F. camptoceras NF.2833	5,000	20,000	+
F. culmorum 1	20	10,000	+
F. culmorum HM-8	1,000	35,000	+
F. graminearum 2	20	5,000	+
F. graminearum NF.1432	25,000	35,000	–
F. graminearum NF.1436	75	10,000	+
F. graminearum NF.0996	10,000	35,000	+
F. moniliforme NF.1439	100	5,000	–
F. moniliforme 248	2,500	5,000	–
F. moniliforme var. *subglutinans* 7	10,000	35,000	–
F. oxysporum 1a	2,000	10,000	–
F. oxysporum 1b	100	5,000	–
F. oxysporum 1c	3,500	10,000	–
F. poae 4	20	5,000	+
F. poae NF.5098	5	100	+, D
F. roseum subsp. *gibbosum*	10,000	35,000	+
F. roseum subsp. *graminearum* 23	10,000	35,000	+
F. roseum subsp. *graminearum* A-2-2	5	750	–
F. roseum subsp. *graminearum* 27	10,000	35,000	–
F. roseum subsp. *graminearum* 28	10,000	35,000	–
F. roseum subsp. *graminearum* 29	15,000	35,000	–
F. semitectum NF.5099	10,000	20,000	–
F. solani IF	5,000	10,000	–
F. sporotrichiodes NF.2832	25,000	50,000	–
F. sporotrichiodes 33-1972	2	50	+
F. sporotrichiodes 33-1979	1	20	+
F. tricinctum NRRL-3299	0.5	1	+, D
G. nivalis 5	25,000	35,000	–
G. nivalis 6	50,000	50,000	–
G. nivalis 7	150	35,000	+
M. verrucaria C.2561	0.75	1	+
M. verrucaria C.2562	0.5	0.5	+
S. atra 82-X	1	10	+
S. bisby C.2560	150	5,000	–
Papulospora sp. 4	200	20,000	–

[a] D, Death; +, definite skin toxicity; –, no detectable toxic effect.

(Table 4) did not correlate significantly (98% confidence level by analysis of variance) with cytotoxicity values obtained with either line with or without logarithmic transformation or with weight gain or loss or food refusal activity in the feeding test. As expected, food refusal activity was an excellent predictor of weight loss ($r = 0.92$, $F = 204$, $P = 10^{-12}$), consistent with food refusal rather than direct toxic effects being responsible for weight loss. Cytotoxicity values obtained with mouse and human cell lines correlated well ($r = 0.70$, $F = 35.1$, $P = 10^{-6}$).

The highly significant correlation between cytotoxicity and food refusal activity is consistent with the cytotoxic agents also possessing food refusal activity. However, there are some isolates that induce much greater weight loss than predicted by the line of best fit in Fig. 2. This observation suggests that isolates such as *F. camptoceras* NF.2833 and *F. roseum* subsp. *graminearum* 23 may produce an agent(s) with strong food refusal activity and weak or no cytotoxicity. Despite these exceptions the results indicate that cytotoxicity assays can be valuable predictors of toxicity in screening fungal isolates for mycotoxin production.

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